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H1

BINDING SUBSTANCES

The present inv ntion r lates to binding substances. In particular, the present invention relates to methods for the production of binding substances eg binding molecules and to the biological binding molecules produced by these methods. In particular, the present invention relates to: a) the production of antibodies, receptor molecules and fragments and derivatives of these antibodies and receptor molecules; b) viruses encoding the above identified molecules which viruses have the ability to present said molecules at their surfaces; c) packages comprising a virus and an above identified molecule presented at the viral surface; and d) screening techniques utilising the unique properties of these packages..

Owing to their high specificity for a given antigen, the advent of monoclonal antibodies (Kohler, G. and Milstein C; 1975 Nature 256: 495) represented a significant technical break-through with important consequences both scientifically and commercially.

Monoclonal antibodies are made by establishing an immortal mammalian cell line which is derived from a single immunoglobulin producing cell secreting one form of a biologically functional antibody molecule with a particular specificity. Because an immortal antibody-secreting mammalian cell line is produced, the characteristics of the antibody are reproducible from batch to batch. The key properties of monoclonal antibodies are their specificity for a particular antigen and the reproducibility with which they can be manufactured.

Structurally, the simplest antibody (IgG) comprises four polypeptide chains, two heavy (H) chains and two light (L) chains inter-connected by disulphide bonds (see figure 1). Each chain has a constant r gion (C) and a variable region (V). The antibody has two arms (the Fab region) each of which has a V_L and a V_H region associated with each other. It is this pair of V regions (V_L and V_H) that differ

from on antibody to anoth r, and which together are responsible for recognising the ntigen. In even more detail, each V region is made up from three complementarity determining regions (CDR) separated by four framework regions (FR). The CDR's are the most variable part of the variable regions, and they perform the critical antigen binding function. The CDR regions are derived from many potential germ line sequences via a complex process involving recombination, mutation and selection.

It has been shown that the function of binding antigens can be performed by fragments of a whole antibody. Binding fragments are the F_v fragment which comprises the V_L and V_H of a single arm of the antibody, and the dAb fragment (Ward, E.S. et al., Nature 341, 544-546 (1989); which consists of a single heavy chain variable domain (V_H).

Although the F_v fragment is coded for by separate genes, it has proved possible to construct a linker that enables them to be made as a single protein chain (known as single chain F_v ($scFv_1$; Bird, R.E. et al., Science 423, 423-426 (1988) Huston, J.S. et al., Proc. Natl. Acad. Sci., USA 85, 5879-5883 (1988)) by recombinant methods.

Whilst monoclonal antibodies, their fragments and derivatives have been enormously advantageous, there are nevertheless a number of limitations associated with them.

Firstly, the therapeutic applications of monoclonal antibodies produced by human immortal cell lines holds great promise for the treatment of a wide range of diseases (Clinical Applications of Monoclonal Antibodies. Edited by E. S. Lennox. British Medical Bulletin 1984. Publishers Churchill Livingstone). Unfortunately, immortal antibody-producing human cell lines are very difficult to establish and they give low yields of antibody (approximately 1 µg/ml). In contrast, equivalent rodent cell lines yield high amounts of antibody (approximately 100 µg/ml). How ver, the repeated administration of these foreign rodent proteins to humans can lead to harmful

hyp rs nsitivity reactions. In the main therefore, these rodent-derived monoclonal antibodies have limited therapeutic use.

Secondly, a key aspect in the isolation of monoclonal antibodies is how many different antibody producing cells with different specificities, can be sampled compared to how many need to be sampled in order to isolate a cell producing antibody with the desired specificity characteristics (Milstein, C., Royal Soc. Croonian Lecture, Proc. R. Soc. London B. 239; 1-16, (1990)). For example, the number of different specificities expressed at any one time by lymphocytes of the murine immune system is thought to be approximately 10^7 and this is only a small proportion of the potential repertoire of specificities. However, during the isolation of a typical antibody producing cell with a desired specificity, the investigator is only able to sample 10^3 to 10^4 individual specificities. The problem is worse in the human, where one has approximately 10^{12} lymphocyte specificities, with the limitation on sampling of 10^3 or 10^4 remaining.

This problem has been alleviated to some extent in laboratory animals by the use of immunisation regimes. Thus, where one wants to produce monoclonal antibodies having a specificity against a particular epitope, an animal is immunised with an immunogen expressing that epitope. The animal will then mount an immune response against the immunogen and there will be a proliferation of lymphocytes which have specificity against the epitope. Owing to this proliferation of lymphocytes with the desired specificity, it becomes easier to detect them in the sampling procedure. However, this approach is not successful in all cases, as a suitable immunogen may not be available. Furthermore, where one wants to produce human monoclonal antibodies (eg for therapeutic administration as previously discussed) such an approach is not practically or ethically feasible.

In the last few years, thes problems have in part,

been addressed by the application of recombinant DNA methods to the isolation and production of antigen binding fragments of an antibody mol cule in bacteria such as E.coli. Furthermore, the use of polymerase chain reaction (PCR) amplification (Saiki, R.K., et al., Science 239, 4387-491 (1988)) to isolate antibody producing sequences from cells and organs, has great potential for speeding up the timescale under which specificities can be isolated. Amplified V_H and V_L genes are cloned directly into vectors for expression in bacteria or mammalian cells (Orlandi, R., et al., 1989, Proc. Natl. Acad. Sci., USA 86, 3833-3837; Ward, E.S., et al., 1989 supra; Larrick, J.W., et al., 1989, Biochem. Biophys. Res. Commun. 160, 1250-1255; Sastry, L. et al., 1989, Proc. Natl. Acad. Sci., USA., 86, 5728-5732). Conversely, some of these techniques can exacerbate the screening problems. For example, large separate heavy and light chain libraries have been produced from immunized mice and combined together in a random combinatorial manner prior to screening (Huse, W.D. et al., 1989, Science 246, 1275-1281). Crucially however, the information held within each cell, namely the specific combination of one light chain with one heavy chain, is lost. This loses most, if not all, of the advantage gained by using immunization protocols in the animal. Currently, only libraries derived from single heavy chain variable domains (dAbs; Ward, E.S., et al., 1989, supra.) do not suffer this drawback, but because not all antibody heavy chain variable regions are capable of binding antigen, more have to be screened.

In addition, the problem of directly screening many different specificities in prokaryotes remains to be solved.

Thus, there is a need for a screening system which ameloriates or overcome one or more of the above or other problems. The ideal system would allow the sampling of very large numbers of specificities (eg of the order of 10^6 and higher) rapid sorting at each cloning round, and rapid

transfer of the genetic material coding for the binding molecule from one stage of the production process, to the next stage.

The most attractive candidates for this type of screening, would be prokaryotic organisms (because they grow quickly, are relatively simple to manipulate and because large numbers of clones can be created) which express and retain antibody on their surface. It has already been shown that antibody fragments can be secreted through bacterial membranes with the appropriate signal peptide (Skerra, A., and Pluckthun, A., 1988, Science 240, 1038-1040; Better, M. et al., 1988, Science 240, 1041-1043). However, it has not been shown how an antibody or antibody fragment can be held on the bacterial cell surface in a configuration which allows efficient sampling of its antigen binding properties. In large part, this is because the bacterial surface is a complex structure, and in the gram-negative organisms there is an outer wall which further complicates the position.

Bacteriophage make attractive candidates because in general their surface is a much simpler structure, they can be grown easily in large numbers, are amenable to the practical handling involved in many potential mass screening programmes and they carry genetic information for their own synthesis within a small, simple package. The difficulty has been to practically solve the problem of how to use bacteriophages in this manner. For example, a Genex Corporation patent application number PCT/US88/00716 has proposed that the bacteriophage lambda would be a suitable vehicle for the expression of antibody molecules, but no proposals provide a teaching which enables the general idea to be carried out. For example PCT/US88/00716 does not demonstrate that any sequences: a) have been expressed as a fusion with gen V; b) have been expressed on the surface of lambda; and c) retain biological activity. Furthermore there is no teaching on how to screen for suitable fusions.

Th problem of how to use bacteriophages is in fact a

difficult one. The antibody molecule must be inserted into the phage in such a way that the integrity of the phage coat is not undermined, and the antibody itself should be biologically active. Thus the antibody should fold efficiently and correctly and be presented for antigen binding. However, solving the problem for antibody molecules and fragments would also provide a general method for the screening of many receptor molecules.

Surprisingly, the applicants have been able to construct a bacteriophage that expresses and presents on its surface large binding molecules (eg large biologically functional antibody molecules) and which remains intact and infectious. The applicants have called the structure which comprises a virus particle and a binding molecule presented at the viral surface a 'package'. Where the binding molecule is an antibody (or a fragment or derivative of an antibody), the applicants call the package a phage antibody. However, except where the context demands otherwise, where the term phage antibody is used generally it should also be interpreted as referring to any package comprising a virus particle and a binding molecule presented at the viral surface.

The present applicants have also been able to develop novel screening systems and assay formats which depend on the unique properties of these packages eg phage antibodies.

The present invention provides a method for producing a package which method comprises the steps of:

a) inserting a nucleotide sequence encoding the binding molecule within a viral genome;

b) culturing the virus containing said nucleotide sequence, so that said binding molecule is expressed by the virus presented at its surface.

The present invention also provides a method for producing a binding molecule specific for a particular epitope which comprises producing a package as described above and the additional step of screening for said binding

molecul by binding of said molecule to said epitope. The method may comprise one or mor of the additional steps of: i) separating the package from the epitope; ii) recovering said package; and iii) using the inserted nucleotide sequence in a recombinant system to produce the binding molecule separate from virus. The screening step may isolate the nucleotide sequence encoding the binding molecule of desired specificity, by virtue of said binding molecule being expressed in association with the surface of the virus.

In the above methods, the binding molecule may be an antibody, or a fragment or derivative of an antibody. Alternatively, the binding molecule may be an enzyme or receptor and fragments/derivatives of any such enzymes or receptors.

In the above methods, the virus may be a filamentous F-specific bacteriophage. The filamentous F-specific bacteriophage may be fd. In particular, it may be a tetracycline resistant version of fd known as fd-tet. The nucleotide sequence may be inserted within the gene III region of fd. The sequence may be inserted after the signal sequence of gene III, preferably after amino acid+1 of the mature protein. The site for insertion may be flanked by short sequences corresponding to sequences which occur at each end of the DNA to be inserted. For example, the protein domain is an immunoglobulin domain, the insertion site in the phage may be flanked by nucleotide sequences which code for the first five amino acids and the last five amino acids of the Ig domain. Such flanking nucleotide sequences are shown in figure 4(2) B and C, wherein the site-flanking nucleotide sequences encode amino acid sequences QVQLQ and VTVSS which occur at either end of the V_H domain, or QVQLQ and LEIKR which occur at either end of the F_V (combined V_H + V_L) domain. Each of these sequences flanking the insertion site may include a suitable cleavage site, as shown in Fig 4.

Alternatively, the flanking nucleotide sequences shown

in figure 4(2) B and C as d scribed abov , may be used to flank the insertion site for any DNA to be inserted, wh ther or not that DNA codes an immunoglobulin.

In the above methods the nucleotide sequences inserted within the viral genome may be derived from eg mammalian spleen cells or peripheral blood lymphocytes. The mammal may be immunised or non-immunised. Alternatively, the nucleotide sequence may be derived by the in vitro mutagenesis of an existing antibody coding sequence. The phage particle presenting said binding molecule may remain intact and infectious.

As previously mentioned, the present invention also provides novel screening systems and assay formats. In these systems and formats the gene sequence encoding the binding molecule (eg the antibody) of desired specificity is separated from the general population having a range of specificities by the fact of its binding to a specific target (eg the antigen or epitope).

Thus, the present invention provides a method of screening a population of phage antibodies (where the binding molecule is an antibody) for a phage antibody with a desired specificity, which comprises contacting said population of phage antibodies with a desired epitope and separating phage antibody which binds to said epitope, from said epitope. The means for separating any binding phage antibodies may be varied in order to obtain binding phage antibodies with different binding affinities for said epitope.

Alternatively, in order to obtain high affinity phage antibodies the epitope may be presented to the population of phage antibodies already with a binding member for said epitope bound thereto, in which case, phage antibodies with a higher binding affinity for said epitope than said bound binding member will displace said bound binding member. The high affinity phage antibodies can then be separated from said epitope.

Separation of phage antibodi s from said epitope may

be achiev d by eg elution techniques well known in the art, infection of suitable bacteria tc.

The present invention lso provides packages as defined above and binding molecules (eg antibodies, enzymes, receptors; fragments and derivatives thereof), obtainable by use of any of the above defined methods, systems and formats.

The applicants have chosen the filamentous F- specific bacteriophages as an example of the type of phage that could provide a vehicle for the expression of antibodies and antibody fragments and derivatives on their surface and facilitate subsequent screening and manipulation.

The F-specific phages (eg f1, fd and M13) have evolved a method of propagation which does not kill the host cell and they are used commonly as vehicles for recombinant DNA (Kornberg, A., DNA Replication, W.H. Freeman and Co., San Francisco, 1980). The single stranded DNA genome (approximately 6.4 Kb) is extruded through the bacterial membrane where it sequesters capsid sub-units, to produce mature virions. These virions are 6 nm in diameter, 1μm in length and each contain approximately 2,800 molecules of the major coat protein encoded by viral gene VIII and four molecules of the adsorption molecule encoded by viral gene III. The latter is located at one end of the virion. The structure has been reviewed by Webster et al., 1978 in The Single Stranded DNA Phages, 557-569, Cold Spring Harbor Laboratory Press. The gene III product is involved in the binding of the phage to the bacterial F-pilus.

Although these phages do not kill their host during normal replication, disruption of some of their genes can lead to cell death (Kornberg, A., 1980 supra.) This places some restraint on their use. The applicants have recognized that gene III of phage fd is an attractive possibility for the insertion of biologically active foreign sequences. The protein its lf is only a minor component of the phage coat and disruption of the gene does not lead to cell death (Smith, G. 1988, Virology 167: 156-

165). Furthermore, it is possible to insert some for ign s quences (with no biological function) into various positions within this gene (Smith, G., 1985 Science 228: 1315-1317., Parmley, S.F. and Smith, G.P Gene: 73 (1988) p. 305-318., and de la Cruz, V.F., et al., 1988, J. Biol. Chem., 263: 4318-4322). In these cases, although the infectivity of the virion was disrupted, the inserted sequences could be detected on the phage surface.

The protein encoded by gene III has several domains (Pratt, D., et al., 1969 Virology 39:42-53., Grant, R.A., et al., 1981, J. Biol. Chem. 256: 539-546 and Armstrong, J., et al., FEBS Lett. 135: 167-172 1981.) including: i) a signal sequence that directs the protein to the cell membrane and which is then cleaved off; ii) a domain that anchors the mature protein into the bacterial cell membrane (and also the phage coat); and iii) a domain that specifically binds to the phage receptor the F-pilus of the host bacterium. Short sequences derived from protein molecules have been inserted into two places within the mature molecule (Smith, G., 1985 supra., and Parmley, S.F. and Smith, G.P., 1988 supra.) into an inter-domain region and also between amino acids 2 and 3 at the N-terminus. The insertion sites at the N-terminus were more successful in maintaining the structural integrity of the gene III protein and displaying the peptides on the surface of the phage. By use of specific antisera, the peptides inserted into this position were shown to be on the surface of the phage. These authors were also able to purify the phage using this property. However, the peptides expressed by the phage, did not possess measurable biological functions of their own.

Retaining the biological function of a molecule when it is expressed in a radically different context to its natural state is difficult. The demands on the structure of the molecule are heavy. In contrast, ret ining the ability to be bound by specific antisera is a passive process which imposes far less rigorous demands on the

structur of the molecule. For example, it is the rule rather than the exception that polyclonal antis ra will recognise totally denatured, and biologically inactive, proteins on Western blots (see for example, Harlow, E. and Lane, D., Antibodies, a Laboratory Manual, Cold Spring Harbor Laboratory Press 1988). Therefore, the insertion of peptides into a region that allows their structure to be probed with antisera teaches only that the region allows the inserted sequences to be exposed and does not teach that the region is suitable for the insertion of large sequences with demanding biological function.

This experience with Western blots is a graphic practical demonstration which shows that retaining the ability to be bound by specific antisera imposes far less rigorous demands on the structure of a molecule, than does the retention of a biological function.

The applicants have investigated the possibility of inserting biologically active antibody fragments into the gene III region of fd to create a large fusion protein. As is apparent from the previous discussion, this approach makes onerous demands on the functionality of the fusion protein. The insertion is large, 100-200 amino acids; the antibody derived domain must fold efficiently and correctly to retain antigen-binding; and most of the functions of gene III must be retained. The applicants approach to the construction of the fusion molecule was designed to minimise the risk of disrupting these functions. The initial vector used was fd-tet (Zacher, A.N., et al., 1980, Gene 9, 127-140) a tetracycline resistant version of fd bacteriophage that can be propagated as a plasmid that confers tetracycline resistance to the infected E.coli host. The applicants chose to insert after the signal sequence of the fd gene III protein for sev ral reasons. In particular, the applicants chose to insert after amino acid 1 of the mature protein to retain the context for the signal peptidase cleavage. To retain the structure and function of gene III itself, the majority of the original

amino acids are synthesized after the inserted immunoglobulin sequences. The inserted immunoglobulin sequences were designed to include residues from the switch region that links V_H-V_L to C_H1-C_L (Lesk, A., and Chothia, C., Nature 335, 188-190, 1988).

Surprisingly, by manipulating gene III of bacteriophage fd, the present applicants have been able to construct a bacteriophage that expresses on its surface large biologically functional antibody molecules and which remains intact and infectious. Furthermore, the phages bearing antibodies of the correct specificity, can be selected from a background where the majority of phages do no show this specificity.

The population of antibody molecules inserted into the phage can be derived from a variety of sources. For example, immunised or non-immunised rodents or humans, and from organs such as spleen and peripheral blood lymphocytes. The coding sequences are derived from these sources by techniques familiar to those skilled in the art (Orlandi, R., et al., 1989 supra; Larrick, J.W., et al., 1989 supra; Chiang, Y.L., et al., 1989 Bio Techniques 7, p. 360-366; Ward, E.S, et al., 1989 supra; Sastry, L., et al., 1989 supra.) Each individual phage antibody in the resulting library of phage antibodies will express antibody derived fragments that are monoclonal with respect to its antigen-binding characteristics.

The disclosure made by the present applicants is important and provides a significant breakthrough in the technology relating to the production of biological binding molecules, their fragments and derivatives by the use of recombinant methods.

In standard recombinant techniques for the production of antibodies, an expression vector containing sequences coding for the antibody polypeptide chains is used to transform eg E.coli. The antibody polypeptides are expressed and detected by use of standard screening systems. When the screen detects an antibody polypeptide

of th desired sp cificity, one has to r turn to the particular transformed E.coli expressing th desired ntibody polyp ptide. Furth rmore, the vector containing the coding sequence for the desired antibody polypeptide then has to be isolated for use from E.coli in the further processing steps.

In the present invention however, the desired antibody polypeptide when expressed, is already packaged with its gene coding sequence. This means that when the screen detects an antibody polypeptide of desired specificity, there is no need to return to the original culture for isolation of that sequence.

Because the phage antibody is a novel structure that contains an antibody of monoclonal antigen-binding specificity on the surface of a relatively simple structure also containing the genetic information encoding its function, phage antibodies that bind antigen can be recovered very efficiently by either eluting off (eg using diethylamine, high salt etc) and infecting suitable bacterial or by denaturing the structure and specifically amplifying the antibody encoding sequences using PCR. That is, there is no necessity to refer back to the original bacterial clone that gave rise to the phage antibody.

Individual phage antibodies expressing the desired antigen-binding specificity can be isolated from the complex library using the conventional screening techniques (eg as described in Harlow, E., and Lane, D., 1988, supra). One example is illustrated in figure 2(i). This shows antigen (ag) bound to a solid surface (s). The population of phage antibodies is then passed over the antigen, and those individuals p that bind are retained after washing, and optionally detected with detection system d. One possible detection system based upon anti-fd antisera is illustrated below in example 4. Since the bound phage antibody can be amplified using for example PCR or bacterial infection, it is also possible to rescue the desired specificity even when insufficient individuals are bound to allow detection via conventional techniques.

The efficiency of this screening procedure for phage antibodies and the ability to create very large libraries means that the immunisation techniques developed to increase the proportion of screened cells producing antibodies of interest will not be an absolute requirement. The technique allows the rapid isolation of antigen-binding specificities, including those that would be difficult or even unobtainable by conventional techniques, for example, catalytic or anti-idiotypic antibodies. Removal of the animal altogether is now possible once a complete library of the immune repertoire has been constructed.

Affinity Maturation Screening Formats

The applicants have also devised a series of novel screening techniques that are practicable only because of the unique properties of phage antibodies. The general outline of some screening procedures is illustrated in figure 2.

The population/library of phage antibodies to be screened could be generated from immunised or other animals; or be created in vitro by mutagenising pre-existing phage antibodies (using techniques well-known in the art such as oligonucleotide directed mutagenesis

(Sambrook, J., et al., 1989 Molecular Cloning a Laboratory Manual, Cold Spring Harbor Laboratory Press). This population can b screened in one or more of the formats described below with reference to figure 2, to derive those individual phage antibodies whose antigen binding properties are different from sample c. Examples of the possible screening formats are:

Binding/Elution

Referring to figure 2(i) population p binds to antigen ag fixed to a solid support s. If samples of bound population p are removed under increasingly stringent conditions, the binding affinity represented in each sample will increase. Conditions of increased stringency can be obtained, for example, by increasing the time of soaking or changing the pH of the soak solution, etc.

Competition

Referring to figure 2(ii) antigen ag can be bound to a solid support s and bound to saturation by the original binding molecule c. If a population of mutant phage antibody (or a set of unrelated phage antibody) p is offered to the complex, only those that have higher affinity for antigen ag than c will bind. In most examples, only a minority of population c will be displaced by individuals from population p. If c is a traditional antibody molecule, all bound material can be recovered and bound p recovered by infecting suitable bacteria and/or by use of standard techniques such as PCR.

An advantageous application is where ag is used as a receptor and c the corresponding ligand. The recovered bound population p is then related structurally to the receptor binding site/and or ligand. This type of specificity is known to be very useful in the pharmaceutical industry.

Another advantageous application is where ag is an antibody and c its antigen. The recovered bound population p is then an anti-idiotype antibody which have numerous uses in research and the diagnostic and pharmaceutical

industries.

In some instances it may prove advantageous to pre-select population p. For example, in the anti-idiotype example above, p can be absorbed against a related antibody that does not bind the antigen.

However, if c is a phage antibody, then either or both c and p can advantageously be marked in some way to both distinguish and select for bound p over bound c. This marking can be physical, for example, by pre-labelling p with biotin; or more advantageously, genetic. For example, c can be marked with an EcoB restriction site, whilst p can be marked with an EcoK restriction site (see Carter, P. et al., 1985, Nucl. Acids Res. 13, 4431-4443). When bound p+c are eluted from the antigen and used to infect bacteria, there is restriction (and thus no growth) of population c (i.e. EcoB restricting bacteria in this example). Any phage that grew, would be greatly enriched for those individuals from p with higher binding affinities. Alternatively, the genetic marking can be achieved by marking p with new sequences, which can be used to specifically amplify p from the mixture using PCR.

The novel structure of the phage antibody molecule can be used in a number of other applications some examples of which are:

Signal Amplification

Acting as a novel molecular entity in itself, phage antibodies combine the ability to bind the specific antigen with the amplification, if the major coat protein is used to attach another moiety. This moiety can be attached via immunological, chemical, or any other means and can be used, for example, to label the complex with detection reagents or cytotoxic molecules for use in vivo or in vitro.

Physical Detection

The size of the phage antibody can be used as a marker particularly with r spect to physical methods of detection such as electron microscopy and/or some biosensors, eg.

surfac plasmon resonance.

Diagnostic Assays

The phag antibody molecule also has advantageous uses in diagnostic assays, particularly where separation can be effected using its physical properties for example centrifugation, filtration etc.

In order that the invention is more fully understood, embodiments will now be described in more detail by way of example only and not by way of limitation with reference to the figures described below.

Figure 1 shows the basic structure of the simplest antibody molecule IgG.

Figure 2 shows schematically novel screening techniques which utilise the unique properties of phage antibodies.

Figure 3 shows a scheme for the construction of vectors.

Figure 4 shows the nucleotide sequences for the oligonucleotides and vectors. All sequences are drawn 5' to 3' and are numbered according to Beck et al., 1978, Nucl. Acid Res., 5: 4495-4503. 4.1 shows the sequences of the ologonucleotides used for mutagenesis (oligo's 1 and 2) or sequencing (oligo 3). The sequences shown were synthesized on an Applied Biosystems, olgonucleotide synthesizer and are complementary to the single stranded form of fd-tet (they are in the anti-sense form with respect to gene III). 4.2 shows the sequences of the various constructs around the gene III insertion site. these sequences are drawn in the sense orientation with respect to gene III; a) fd-tet (and FDTdBst) b) FDTPs/Bs and c) FDTPs/Xh. The key restriction enzyme sites are shown along with the immunoglobulin amino acids contributed by the vectors, (amino acid single letter code is used, see Harlow, E., and Lane, D., 1988 supra.).

Figure 5 shows the nucleotide and amino acid sequences for scFv in the vector scFvD1.3 myc. This gives the

sequence of the anti-lysozym single chain Fv and surrounding sequ nces in scFvD1.3 myc showing the N-terminal pel B signal peptid sequence and the C-terminal myc tag sequence (Ward, E.S., et al., 1989, supra.). Also highlighted is the peptide sequence linking the V_H and V_L regions. The amino acid sequence is represented above the nucleotide sequence by the single letter code, see Harlow, E., and Lane, D., 1988 supra.

Figure 6 shows the effect of varying the amount of supernatant on the binding of phage antibodies to lysozyme in graphical form. Each point is the average of duplicate samples. Lysozyme was coated at 1 mg/ml in 50 mM $NaHCO_3$.

Figure 7 shows the effect of varying the coating concentration on the binding of phage antibodies to lysozyme in graphical form. Each point is the average of duplicate samples. Lysozyme was coated with the specified concentration of either BSA or lysozyme.

The following procedures used by the present applicants are described in Sambrook, J. et al., 1989, supra.: restriction digestion, ligation, preparation of competent cells (Hanahan method), transformation, analysis of restriction enzyme digestion products on agarose gels, purification of DNA using phenol/chloroform, 5'-end labelling of oligonucleotides, filter screening of bacterial colonies, preparation of 2xTY medium and plates, preparation of tetracycline stock solutions, PAGE of proteins, preparation of phosphate buffered saline.

All enzymes were supplied by New England Biolabs (CP Laboratories, PO Box 22, Bishop's Stortford, Herts., England) and were used according to manufacturer's instructions unless otherwise stated.

The vector fd-tet (Zacher, A.N et al., 1980, supra.) was obtained from the American Type Culture Collection (ATCC No. 37000) and transform d into competent TG1 cells (genotype: K12δ (lac-pro), sup E, thi, hsdD5/F'traD36, pro A+B+, Lac I^q, lac δM15).

Viral particles were prepared by growing TG1 cells

containing the desired construct in 10 to 100 mls 2xTY medium with 15 µg/ml t tracycline for 16-24 hours. The culture supernatant was collected by centrifugation for 10 mins at 10,000 rpm in an 8 x 50 ml rotor, Sorval RC-5B centrifuge. Phage particles were precipitated by adding 1/5th volume 20% polyethylene glycol (PEG)/2.5M NaCl and leaving at 4°C for 1 hour. These were spun for 15 minutes as described above and the pellets resuspended in 10 mM Tris/HCl pH 8, 1 mM EDTA to 1/100th of the original volume. Residual bacteria and undissolved material were removed by spinning for 2 minutes in a microcentrifuge. Single stranded DNA for mutagenesis or sequencing was prepared from concentrated phage according to Sambrook, J., et al., 1989, supra.

Example 1

Design of Insertion Point Linkers and Construction of Vectors

The vector fd-tet has two BstEII restriction sites flanking the tetracycline resistance gene (fig 3). Since the strategy for inserting the V_H fragments was to ligate them into a newly inserted BstEII site within gene III, it was advantageous to delete the original BstEII sites from fd-tet. This was achieved by digesting fd-tet with the restriction enzyme BstEII, filling-in the 5' overhangs and re-ligating to generate the vector FDT6Bst. Digestion of fd-tet with BstEII (0.5 units/µl) was carried out in 1x KGB buffer (100 mM potassium glutamate, 25 mM Tris-acetate (PH 7.5), 10 mM magnesium acetate, 50 µg/ml bovine serum albumin, 0.5 mM (Sambrook, J., et al., 1989, supra.) with DNA at a concentration of 25 ng/µl. The 5' overhang was filled in, using 2x KGB buffer, 250 µM each dNTP's (Pharmacia Ltd., Pharmacia House, Midsummer Boulevard, Milton Keynes, Bucks., UK.) and Klenow Fragment (Amersham International, Lincoln Place, Green End, Aylesbury, Bucks., UK) at 0.04 units/ µl. After incubating for 1 hour at room temperature, DNA was extracted with ph nol/chloroform and precipitated with ethanol.

Ligations wer carried out at a DNA concentration of 50ng/µl for 1 hour at room temperature using T4 DNA ligase (40 units/µl). Ligations were transformed into competent TG1 cells and plated onto TY plates supplemented with 15 µg/ml tetracycline. Colonies were picked into 25 mls of 2xTY medium supplemented with 15 µg/ml tetracycline and grown overnight at 37°C.

Double stranded DNA was purified from the resulting clones using the gene-clean II kit (Bio101 Inc., PO Box 2284, La Jolla, California, 92038-2284, USA.) and according to the small scale rapid plasmid DNA isolation procedure described therein. The orientation of 5 of the resulting clones was checked using the restriction enzyme ClaI. A clone was chosen which gave the same ClaI pattern as fd-tet, but which had no BstE II sites.

In vitro mutagenesis of FDT6Bst was used to generate vectors that facilitated cloning of antibody fragments downstream of the gene III signal peptide and in frame with the gene III coding sequence. The oligonucleotide directed mutagenesis system, version 2 (Amersham International) was used with oligo 1 (figure 4) to create FDTPs/Bs. The sequence of FDTPs/Bs (figure 4) was confirmed using the sequenase version 2.0 kit (USB Corp., PO Box 22400, Cleveland, Ohio, 44122, USA.) with oligo 3 (figure 4) as a primer.

A second vector FDTPs/Xh (to facilitate cloning of single chain F_v fragments) was generated by mutagenising FDTPs/Bs with oligo 2 according to the method of Venkitaraman, A.R., Nucl. Acid Res. 17, p 3314. The sequence of FDTPs/Xh (figure 4) was confirmed using the sequenase version 2.0 kit (USB Corp.) with oligo 3 as a primer.

Clearly, alternative constructions will be apparent to those skilled in the art. For exampl , M13 and/or its host bacteria could be modified such that its gene III could be disrupted without the onset of excessive cell death; the modified fd gene III, or other modified prot in, could be

incorporated into a plasmid containing a singl stranded phage replication origin, such as pUC119, superinfection with modifi d phage such as K07 would then result in the encapsulation of the phage antibody genome in a coat partially derived from the helper phage and partly from the phage antibody gene III construct.

The detailed construction of a vector such as FDTPs/Bs is only one way of achieving the end of a phage antibody. For example, techniques such as sticky feet cloning/mutagenesis (Clackson, T. and Winter, G. 1989 Nucl. Acids. Res., 17, p 10163-10170) could be used to avoid use of restriction enzyme digests and/or ligation steps.

Example 2.

Insertion of Immunoglobulin F_v Domain into Phage Antibody

The plasmid scFv D1.3 myc (gift from G. Winter and A. Griffiths) contains V_H and V_L sequences from the antibody D1.3 fused via a peptide linker sequence to form a single chain F_v version of antibody D1.3. The sequence of the scF_v and surrounding sequences in scFvD1.3 myc is shown in figure 5.

The D1.3 antibody is directed against hen egg lysozyme (Harper, M. et al., 1987, Molec. Immunol. 24, 97-108) and the scFv form expressed in E. coli has the same specificity (A. Griffiths and G. Winter personal Communication).

Digestion of scFv D1.3 myc with PstI and XhoI, excises a fragment of 693 bp which encodes the bulk of the scFv. Ligation of this fragment into FDTPs/Xh cleaved with PstI and XhoI gave rise to the construct FDTSCFVD1.3 encoding the gene III signal peptide and first amino acid fused to the complete D1.3 scFv, followed by the mature gene III protein from amino acid 2.

The vector FDTPs/Xh was prepared for ligation by digesting with the PstI and XhoI for 2 hours followed by digestion with calf intestinal alkalin phosphatase (Boehringer Mannheim UK Ltd., Bell Lane, Lewes, East Sussex, BN7 1LG) at one unit/ul for 30 minutes at 37°C. Fresh calf intestinal alkaline phosphatase was added to a

fin 1 total concentration of 2 units/ul and incubated for a further 30 minutes at 37°C. The reaction was extracted three times with phenol/chloroform, precipitated with ethanol and dissolved in water. The insert from scFvD1.3 myc was excised with the appropriate restriction enzymes, extracted twice with phenol/chloroform, precipitated with ethanol and dissolved in water. Ligations were carried out as described in example 1 except both vector and insert samples were at a final concentration of 5 ng/ul each. The formation of the correct construct was confirmed by sequencing as described in example 1.

To demonstrate that proteins of the expected size were produced, virions were concentrated by PEG precipitation as described above, and the equivalent of 2mls of supernatant was loaded onto an 18% SDS polyacrylamide gel. After electrophoresis, the gel was soaked in gel running buffer (50 mM Tris, 380 mM Glycine, 0.1%SDS) with 20% methanol for 15 minutes. Transfer to nitrocellulose filter was executed in fresh 1x running buffer/20% methanol using TE70 Semi Phor a semi-dry blotting apparatus (Hoeffer, 654 Minnesota Street, Box 77387, San Francisco, California 94107, USA.).

After transfer, the filter was blocked by incubation for 1 hour in a 2% solution of milk powder (Cadbury's Marvel) in phosphate buffered saline (PBS). Detection of F_v and V_H protein sequences in the phage antibody fusion proteins was effected by soaking the filter for 1 hour with a 1/1000 dilution (in 2% milk powder) of a rabbit polyclonal antiserum raised against affinity purified, bacterially expressed F_v fragment (gift from G. Winter). After washing PBS (3 x 5 minute washes), bound primary antibody was detected using an anti-rabbit antibody conjugated to horseradish peroxidase (Sigma, Fancy Road, Poole, Dorset, BH17 7NH, UK.) for 1 hour. The filter was washed in PBS/0.1% triton X-100 and dev loped with 0.5 mg/ml 3,3'-diaminobenzidine tetrahydrochloride (DAB), 0.02% cobalt chloride, 0.03% hydrogen peroxide in PBS.

The results show that with FDTVHD1.3 (from example 3)

and FDTSCVFVD1.3, a protein of betwe n 69,000 and 92,500 daltons is det cted by the anti-F_v s rum. This is the expect d size for the fusion proteins constructed. This product is not observed in supernatants derived from fd-tet, FDTδBst or FDTPs/Xh.

Example 3.

Insertion of Immunoglobulin V_H Domain into Phage Antibody

The VH fragment from D1.3 was generated from the plasmid pSW1-VHD1.3-TAG1 (Ward, E.S. et al., 1989 supra.). Digestion with Pst1 and BstEII generates the fragment shown between positions 113 and 432 in figure 5. Cloning of this fragment into the Pst1 and BstEII sites of FDTPs/Bs gave rise to the construct FDTVHD1.3 which encodes a fusion protein with a complete V_H inserted between the first and third amino acids of the mature gene III protein (amino acid two has been deleted).

The methods used were exactly as in example 2 except that the vector used was FDTPs/Bs digested with Pst1 and BstEII.

Example 4.

Analysis of Binding Specificity of Phage Antibodies

The binding of the various phage antibodies to the specific antigen, lysozyme, was analysed using ELISA techniques. Phage antibodies were grown in E.coli and phage antibody particles were precipitated with PEG as in the materials and methods. Bound phage antibody particles were detected using polyclonal rabbit serum raised against the closely related phage M13.

ELISA plates were prepared by coating 96 well plates (Falcon Microtest III flexible plate. Falcon: Becton Dickinson Labware, 1950 Williams Drive, Oxnard, Californa, 93030, USA.) with 200 ul of a solution of lysozyme (1mg/ml unless otherwise stated) in 50 mm $NaHCO_3$ for 16-24 hours. Before use, this solution was removed, the plate rinsed several time in PBS and incubated with 200 ul of 2% milk powder/PBS for 1 hour. After rinsing several times with PBS, 100 ul of the test samples were added and incubated

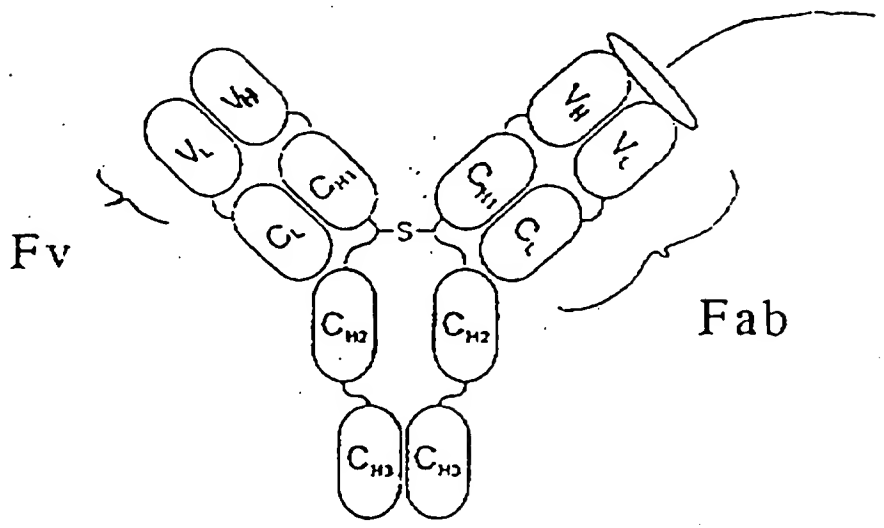
for 1 hour. Plates were washed (3 rinses in 0.05% Tween 20/PBS followed by 3 rinses in PBS alone). Bound phage antibodies were detected by adding 200 ul/w ll of a 1/1000 dilution of sheep anti-M13 polyclonal antiserum (gift from G. Winter) in 2% milk powder/PBS and incubating for 1 hour. After washing as above, plates were incubated with biotinylated anti-sheep antibody (Amersham International) for 30 minutes. Plates were washed as above, and incubated with streptavidin-horseradish peroxidase complex (Amersham International). After a final wash as above, 0.5 mg/ml ABTS substrate in citrate buffer was added (ABTS=2'2'-azinobis(3-ethylbenzthiazoline sulphonic acid); citrate buffer =50 mM citric acid, 50 mM tri-sodium citrate at a ratio of 54:46). Hydrogen peroxide was added to a final concentration of 0.003% and the plates incubated for 1 hour. The optical density at 405 nm was read in a Titertek multiskan plate reader.

Figure 6 shows the effect of varying the amount of phage antibodya. 100 ul of various dilutions of PEG precipitated phage were applied and the amount expressed in terms of the original culture volume from which it was derived. Signals derived from both the scFv containing phage antibody (FDTSCFVD1.3) and the V_H containing phage antibody (FDTVHD1.3) were higher than that derived from the phage antibody vector (FDTPs/Xh). The highest signal to noise ratio occurs using the equivalent of 1.3 mls of culture.

Figure 7 shows the results of coating the plates with varying concentrations of lysozyme or bovine serum albumin (BSA). The equivalent of 1 ml of the original phage antibody culture supernatant was used. The signals from supernatants derived from FDTSCFVD1.3 were again higher than those derived from FDTPs/Xh when lysozyme coated wells were used. There was no significant difference between these two types of supernatant when the plates were coated with BSA. Broadly speaking the level of signal on the plates is proportional to the amount of lysozyme co ted.

These results demonstrate that the binding detected is specific for lysozyme as the antigen.

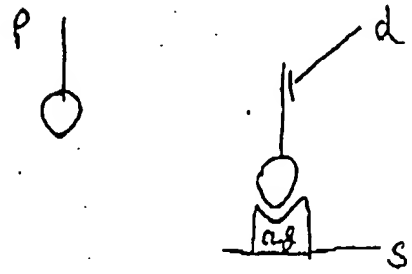
FIGURE 1: Antibody structure



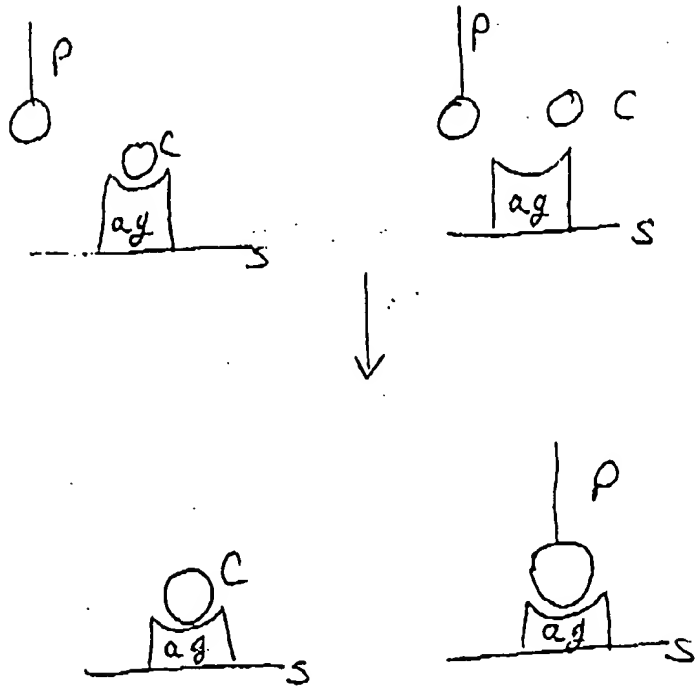
The single domain antibody (dAb, Ward et al. 1989) consists of a single V_H domain.

FIGURE 2: ASSAY FORMATS

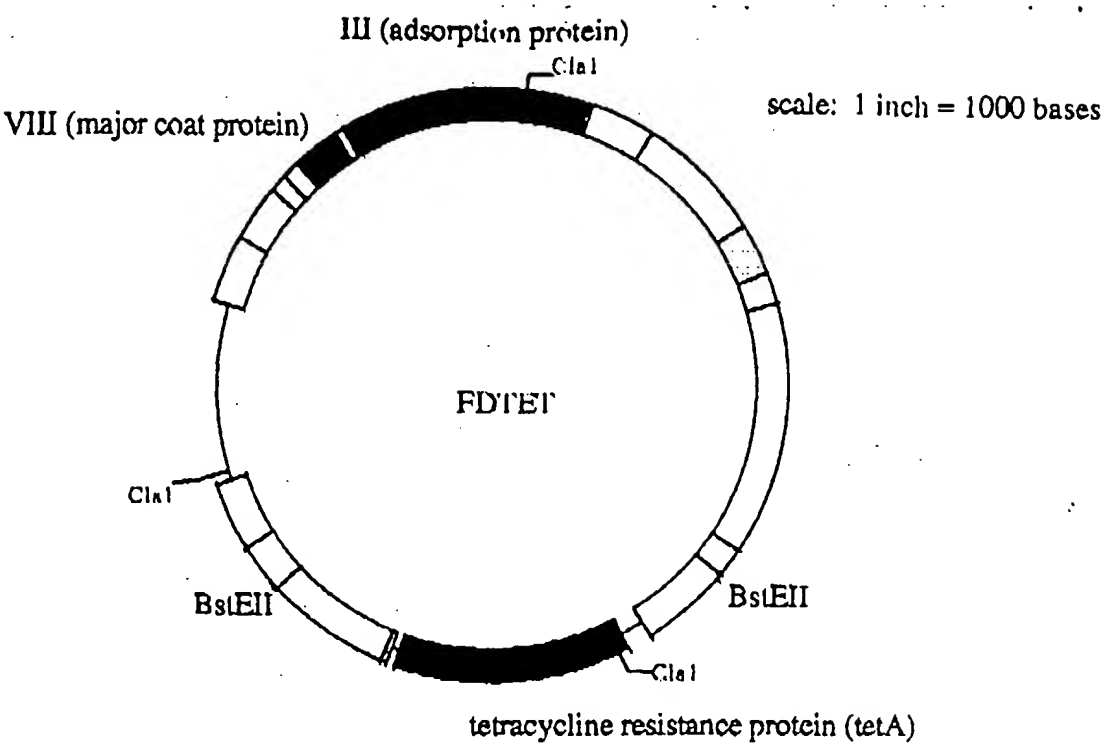
2 i) Binding/elution



2 ii) Compction



p - Phage antibody population to be sampled.
ag - Antigen to which binding required.
c - Competitor antibody/ phAb/ligand etc population.
s - Surface (eg plastic, beads etc).
d - Detection system



fd-tet

⇓

cleave with BstEII

⇓

fill in with Klenow

⇓

re-ligate

▼

FDTδBst

⇓

in vitro mutagenesis (oligo1)

▼

FDTPs/Bs

⇓

in vitro mutagenesis (oligo 2)

▼

FDTPs/Xh

Figure 3 Scheme for construction of vectors

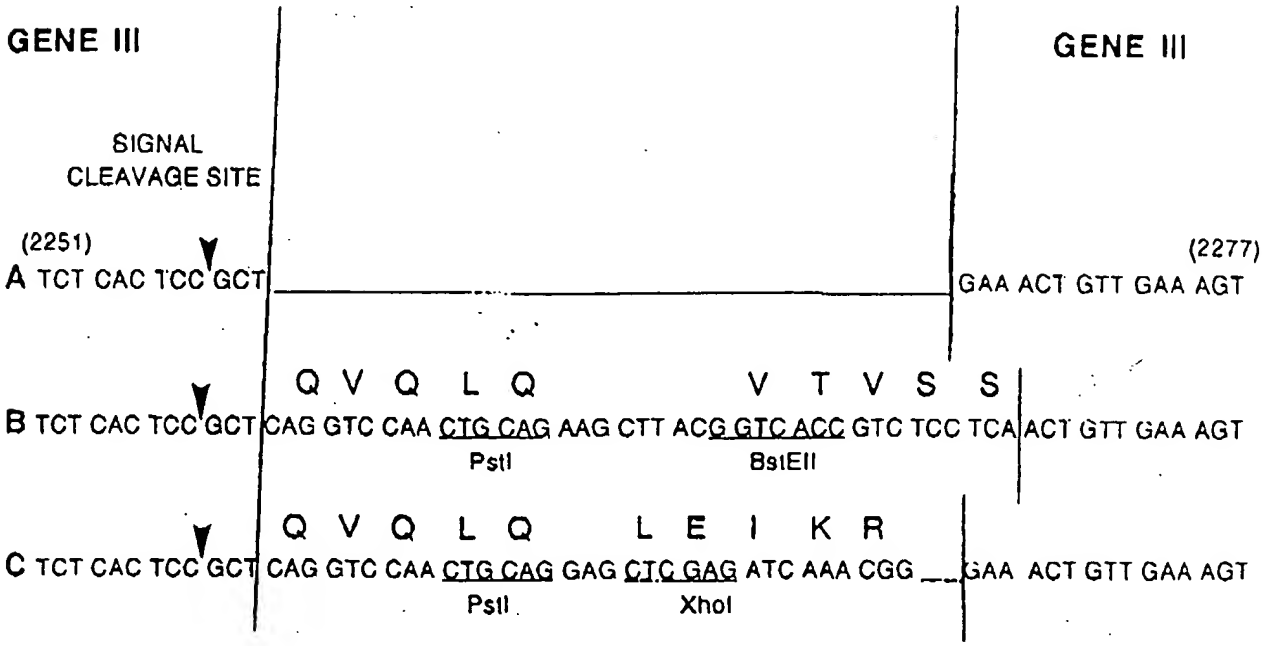
1

(2280)
Oligo 1 ACA ACT TTC AAC AGT TGA GGA GAC GGT GAC CGT AAG CTT CTG CAG TTG GAC CTG AGC GGA GTG AGA ATA (2248)

(2280)
Oligo 2 ACA ACT TTC AAC AGT TTC CCG TTT GAT CTC GAG CTC CTG CAG TTG GAC CTG

(2330)
Oligo 3 GTC GTC TTT CCA GAC GTT AGT

2



B = FDTPs/Bs
C = FDTPs/Xh

Figure 4. Sequence of oligos and vectors

Figure 5. Sequence of SCFvD1.3myc

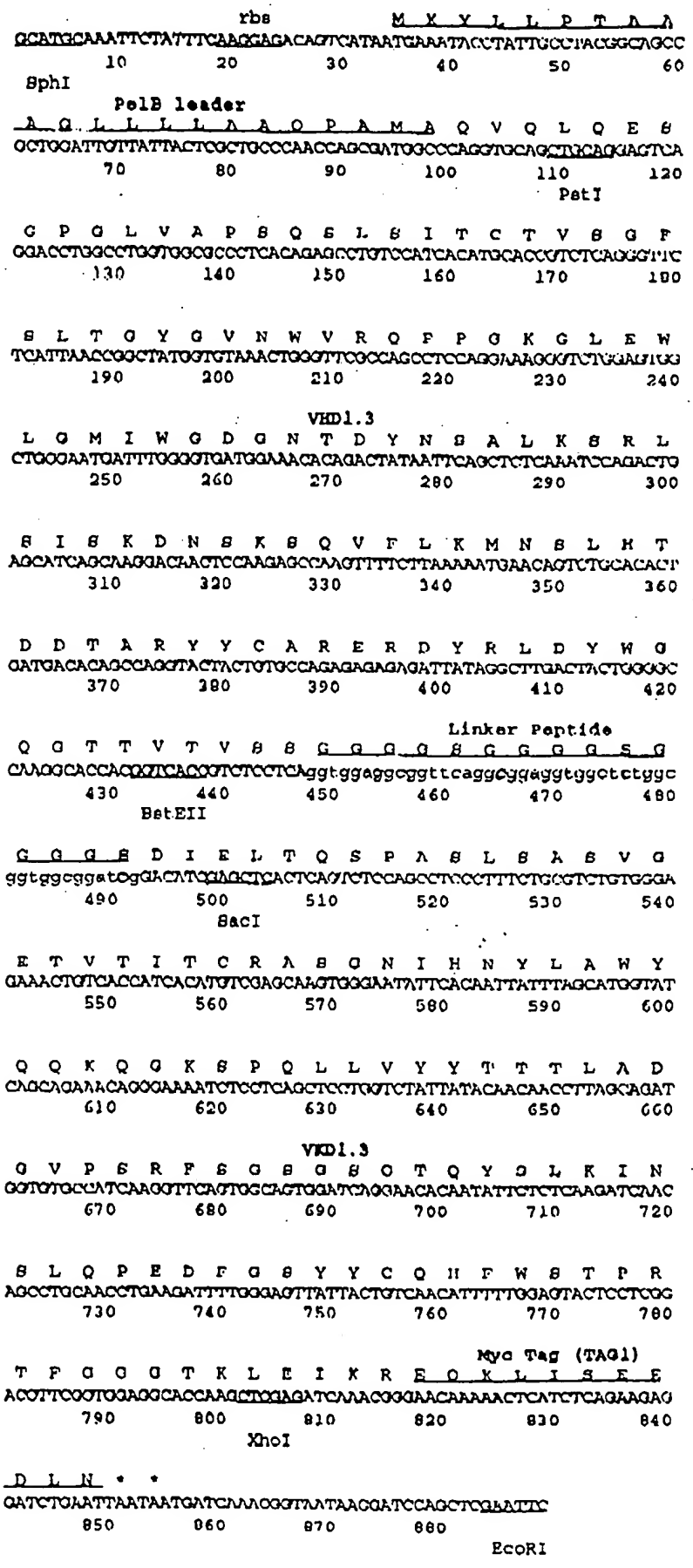
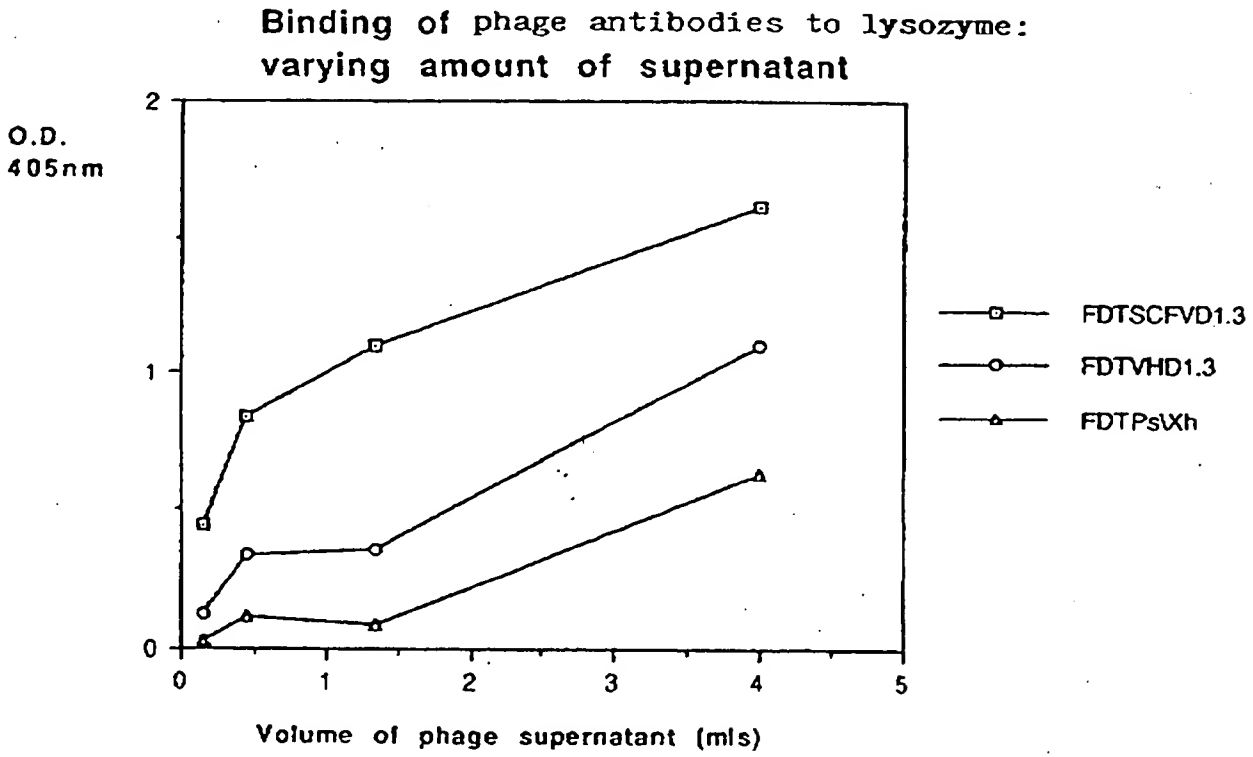
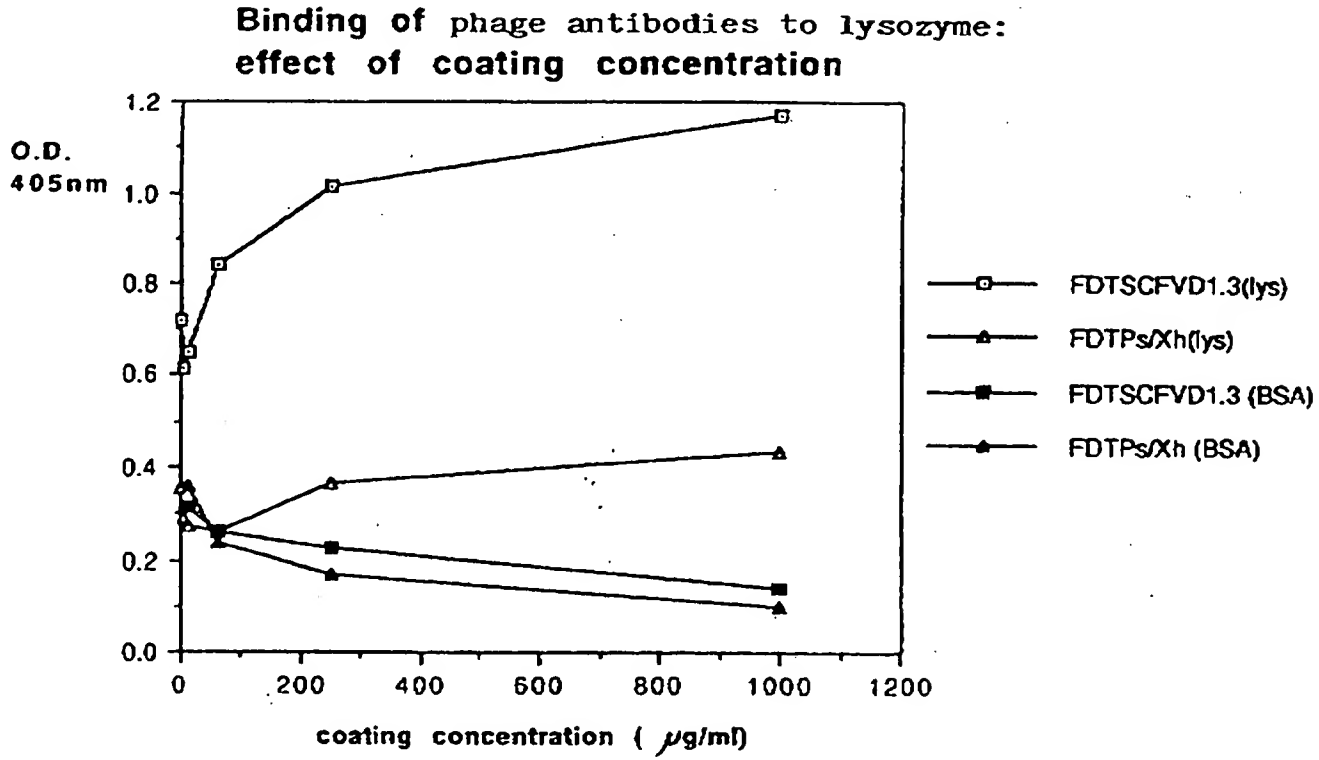


Figure 6



Methods as described in example 4

Figure



Methods as described in example 4

A[illegible] [illegible] [illegible]

5 [illegible] [illegible]

BROAD [illegible]

[illegible]

[illegible]

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